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Quantitative Dot-Immunobinding Assay for Proteins Using Nitrocellulose Membrane Filters

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16. ABSTRACT

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A quantitative dot-immunobinding assay for proteins using nitrocellulose membrane filters

(synapsin I/synaptic vesicles/immunoassay)

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Contributed by Paul Greengard, December 1, 1983

ABSTRACT **An immunoassay method is described for the quantitative determination of synapsin I (protein I) and of a 36,000-dalton membrane protein from rat brain synaptic vesicles. The samples are spotted on nitrocellulose membrane filters, incubated sequentially with specific antibodies and ^{125}I-labeled protein A, and assayed for radioactivity in a gamma scintillation counter. Conditions have been established to prevent losses of protein from the sheets during processing, to quench background radioactivity, and to adjust the sensitivity to the range desired. A large number of samples can be handled in parallel. The assay does not require iodination of the antigen and is accurate even with crude tissue samples. Standard curves were linear over a 20- to 50-fold range. The sensitivity of the method is such that 10 pmol of synapsin I and 50 ng of total vesicle membrane protein could be measured with accuracy. The method should prove useful for a wide range of proteins.**

Nitrocellulose membrane filters have been widely used in molecular biology to immobilize nucleic acids. In recent years it has been found that this material also binds proteins with high efficacy. The immobilized proteins can be analyzed by subsequent binding of specific antibodies and appropriate detection molecules. Iodinated protein A as well as radiolabeled or enzyme-linked anti-antibodies have been used to generate a signal at the binding site (for review see ref. 1). A main application has been the electrophoretic transfer of proteins from gel electropherograms to nitrocellulose membranes ("immunoblotting") (1–4). Direct spotting of small amounts of antigens has also been used for screening of antibodies (5) and for determination of antibody specificity (6). However, there appear to have been no reports of the use of these membrane filters as the basis for a quantitative assay of bound antigens by an antibody-based detection system. In this study we describe a simple and convenient method, based on binding of antigen to nitrocellulose as solid support, for the direct quantitation of two neuronal proteins. The two proteins are: synapsin I (previously referred to as protein I), a neuron-specific phosphoprotein associated with synaptic vesicles (7–10), and a 36,000-dalton intrinsic membrane protein from synaptic vesicles.

MATERIALS AND METHODS

Materials. Nitrocellulose membranes (pore size, 0.22 μm) were obtained from Schleicher & Schuell. ^{125}I-labeled protein A (70–100 μCi/μg; 1 Ci = 37 GBq) was from New England Nuclear, and bovine serum albumin (IgG-free) was from Sigma. All other reagents were of analytical grade and from commercial sources. Synapsin I (protein I), prepared from bovine brain by a modification of the procedure of Ueda and Greengard (11), rabbit antiserum raised against purified synapsin I, and radioiodinated synapsin I were gifts of Jesse Chan, Wilson Wu, and Eva Perdahl, respectively.

Preparation of Antiserum Against Vesicle Membrane Proteins. Synaptic vesicles were purified from rat brain homogenate by the procedure of Huttner *et al.* (10) through the final purification step of controlled-pore glass chromatography. The pooled vesicle-containing fractions (20–40 μg of protein per ml) were added to an equal volume of 0.4 M NaCl in order to dissociate synapsin I (10). After 1 hr on ice the vesicles were pelleted for 2 hr in a Beckman 50 Ti rotor at 50,000 rpm (165,000 × g_{av}). The supernatant that contained synapsin I was discarded. The pellets (stripped vesicles) were resuspended in 300 mM glycine/5 mM Hepes, pH 7.2. Two 300-μg portions of protein were dissolved in 0.1% Triton X-100 (vol/vol), emulsified in complete Freund's adjuvant, and injected intradermally at multiple sites into New Zealand female rabbits. The rabbits were given booster injections with 150 μg of protein at 3 weeks and at 6 weeks after the first injection and bled 2 weeks later.

Standard Procedure for the Dot-Immunobinding Assay. Samples were diluted in sample buffer [120 mM KCl/20 mM NaCl/2 mM $NaHCO_3$/2 mM $MgCl_2$/5 mM Hepes, pH 7.4/0.7% Triton X-100 (vol/vol)]. When $NaDodSO_4$ was used to dissolve crude tissue fractions, the amount of Triton X-100 present in the sample buffer was adjusted so that the concentration of Triton X-100 (vol/vol) was in 7-fold excess over that of $NaDodSO_4$ (wt/vol). A grid of squares (1.8 × 1.8 cm) was drawn on nitrocellulose membrane filters with a soft pencil. Sheets with 54 (9 × 6) squares were used. However, other sizes have been used effectively. The sheets were rinsed for 5 min in distilled water and air-dried immediately before use. Each sample was adjusted to a spotting volume of 20 μl and spotted in three 6- to 7-μl portions on the center of a square, using a hair dryer for drying between the applications. The diameter of the spots was 1.2–1.5 cm. After drying, the sheets could be stored at room temperature. All subsequent steps were carried out at room temperature on a rocking platform using 60 ml of the solution indicated. The sheets were fixed for 15 min in 10% (vol/vol) acetic acid/25% (vol/vol) isopropyl alcohol, rinsed several times with distilled water, and preincubated for 5 min in Tris-buffered saline [200 mM NaCl/50 mM Tris·HCl, pH 7.4 (at 25°C)]. The sheets were then incubated for 1 hr in "blocking solution," consisting of either 5% (wt/vol) bovine serum albumin or 0.5% (wt/vol) gelatin dissolved in Tris/NaCl, in order to reduce nonspecific binding. The sheets were then incubated for 2 hr in antiserum solution [dilution to 1/200th to 1/500th of the antiserum in blocking solution containing 0.1% (vol/vol) Triton X-100], rinsed with Tris/NaCl five times for 5 min each to remove excess IgG, and again incubated in blocking solution for 30 min. The sheets were then incubated for 1 hr in protein A solution [diluted in blocking solution containing 0.1% (vol/vol) Triton X-100 to ≈200,000

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Abbreviation: SGV, synaptic vesicles purified through the sucrose gradient step.

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cpm/ml] and washed in Tris/NaCl/0.1% Triton X-100 four times for 5 min. The sheets were then washed either four times for at least 20 min each (when bovine serum albumin was used as blocking agent) or four times for at least 2 hr each (when gelatin was used as blocking agent) in Tris/NaCl/0.1% Triton X-100. The sheets were finally dried, cut, and assayed for radioactivity in a gamma scintillation counter.

Other Methods. Crude synaptosomes (P_2) and synaptic vesicles purified through the sucrose gradient step (SGV) were obtained as intermediate fractions during the standard vesicle preparation (10). $NaDodSO_4$/polyacrylamide gel electrophoresis was performed according to ref. 12 and radioimmunolabeling of the gel strips was performed by a modification (10) of the method of Adair *et al.* (13). Protein was determined according to Bradford (14). All curves composed of more than five single points were fitted by using a standard linear regression program.

RESULTS

Estimation of Synapsin I and the 36,000-Dalton Synaptic Vesicle Protein Under Standard Conditions. The amount of radioactivity bound in the standard assay for synapsin I, as a function of the amount of synapsin I present in the assay sample, is shown in Fig. 1. A linear relationship was obtained between 10 and 500 fmol of synapsin I. The amount of radioactivity bound in the presence of 10 fmol of synapsin I was approximately three times that of the background.

Proportionality was obtained between the amount of radioactivity bound and the amount of synaptic vesicle protein in the standard assay using anti-vesicle antiserum, as shown in Fig. 2 *Left*. The sensitivity of the assay was dependent on the concentration of antiserum used. Lower concentrations of serum decreased the sensitivity (Fig. 2 *Left*) but extended the linear range to higher values (not shown). In the presence of a 1:500 serum dilution, a linear relationship was obtained between 0.05 and 1 μg of total synaptic vesicle protein. The amount of radioactivity bound in the presence of 0.05 μg of total synaptic vesicle protein was approximately three times that of the background. As can be seen in Fig. 2 *Right*, the antiserum raised against synaptic vesicles was directed predominantly (>95%) against one broad protein band of

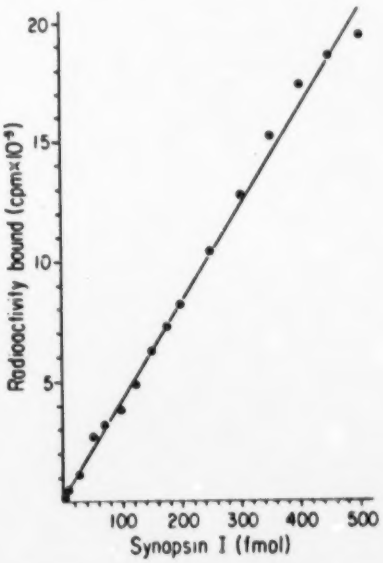

FIG. 1. Effect of various amounts of synapsin I on radioactivity recovered in standard synapsin I assay. The serum dilution was 1:200.

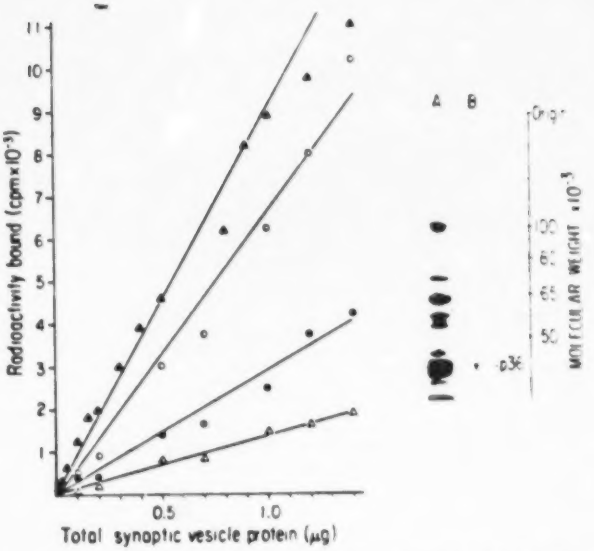

FIG. 2. Effect of various amounts of synaptic vesicle protein on radioactivity recovered in standard assay for 36,000-dalton synaptic vesicle protein. (*Left*) The influence of various dilutions of anti-vesicle antiserum on the calibration curve are shown. The serum dilutions were: ▲, 1:500; △, 1:1000; ●, 1:2000, and ○, 1:4000. (*Right*) Electropherograms of synaptic vesicle proteins (112 μg per lane), separated by $NaDodSO_4$ gel electrophoresis on 7.5% gel: protein staining with Coomassie blue (lane A) and autoradiograph (lane B) obtained after radioimmunolabeling. p36, 36,000-dalton protein.

≈36,000 daltons. No cross reactivity with synapsin I was observed.

Estimation of Synapsin I and the 36,000-Dalton Synaptic Vesicle Protein in Crude Preparations. The amount of synapsin I present in a homogenate of rat brain, determined by the standard assay for synapsin I, was proportional to the amount of brain homogenate assayed (Fig. 3*a*). Linearity was also observed when brain homogenate was assayed for the 36,000-dalton synaptic vesicle protein band (Fig. 3*b*). Addition of various amounts of homogenate to the standard assays for synapsin I and for the 36,000-dalton synaptic vesicle protein indicated excellent recovery of internal standards. Thus, the slopes of the curves relating radioactivity bound to synapsin I (Fig. 4*a*) or synaptic vesicle protein (Fig. 4*b*) were not affected by the addition of crude homogenate. The application of the standard procedure to the assay of synapsin I in a few selected subcellular fractions is shown in Fig. 5. The amount of radioactivity bound was proportional to the amount of subcellular fraction assayed for each of the subfractions. From the slopes of the curves shown in Fig. 5, the relative concentrations of synapsin I per mg of protein in the four subfractions were calculated to be: homogenate, 1.0; P_2, 2.38; SGV, 10.9; and peak fraction of the eluate from controlled pore glass chromatography, 23.0. These relative concentrations are in excellent agreement with those determined by radioimmunoassay (10).

Factors Affecting the Assay. Triton X-100 was found to be essential in the final washing steps to reduce background radioactivity to an acceptable level. Following the standard procedure, background radioactivity varied between 300 and 900 cpm, mainly dependent on the antiserum used. Without Triton in the final washing steps, 5- to 15-fold higher background values were obtained. Although bovine serum albumin and gelatin were each effective in preventing nonspecific binding, more thorough washings were required after the protein A incubation step when gelatin was used as the blocking agent. Extending the time of incubation with blocking solution to 2 hr at 40°C reduced the background further

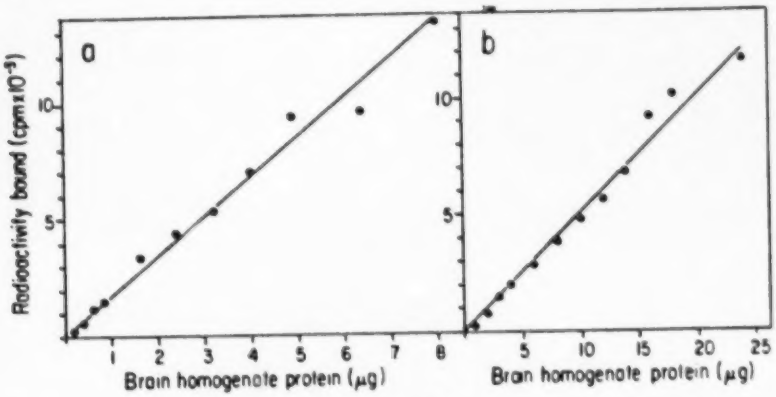

FIG. 3. Proportionality between amount of either synapsin I (*a*) or 36,000-dalton synaptic vesicle protein (*b*), determined by standard assay procedure, and amount of homogenate assayed. The homogenate was dissolved in 1% $NaDodSO_4$ and then diluted with appropriate concentrations of $NaDodSO_4$ and Triton X-100 to achieve final concentrations of 0.1% $NaDodSO_4$/0.7% Triton X-100 prior to application to nitrocellulose sheets.

but led to some loss of protein from the sheets, especially when tissue samples rather then purified standards were tested.

It is essential for quantitation that loss of bound antigen from the nitrocellulose sheets does not occur in the various incubation and washing steps. Using radioiodinated synapsin I, we tested several modifications of the standard procedure. If fixation at the beginning was omitted, synapsin I was washed away to a significant extent. [This loss of protein did not occur if Triton X-100 was omitted from the incubation medium, consistent with an earlier report (4)].

To test crude tissue samples it was often found desirable to dissolve the material in a detergent stronger than the 0.7% Triton X-100 used in the sample buffer. When samples were solubilized in $NaDodSO_4$ (even in concentrations as low as 0.05%) and spotted without further treatment, up to 90% loss of material occurred. However, addition of Triton X-100 to the $NaDodSO_4$-solubilized tissue sample, in a 7-fold excess compared to $NaDodSO_4$, prevented the loss completely (up to 1% $NaDodSO_4$/7% Triton X-100). Therefore, tissue samples were routinely dissolved in 0.1–1% (wt/vol) $NaDodSO_4$, depending on their protein concentration, and thereafter diluted with appropriate amounts of Triton X-100.

The antibody-containing solution could be reused, but this usually led to a reduction in sensitivity. Similar results were obtained with protein A solutions. In general, protein A solutions were re-used up to six times, the cpm/ml being readjusted after each incubation. For storage times of <1 week

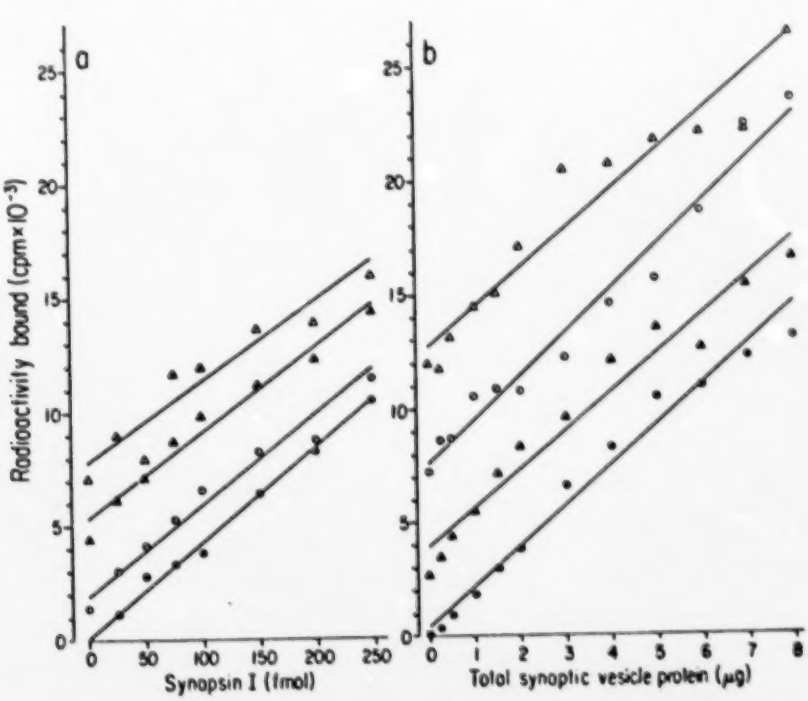

FIG. 4. Effect of various amounts of rat brain homogenate on recovery on either synapsin I (*a*) or 36,000-dalton synaptic vesicle protein (*b*), determined by standard assay procedure. (*a*) No homogenate (●), 0.8 μg of homogenate (○), 2.4 μg of homogenate (▲), or 4 μg of homogenate (△) was added to each sample. (*b*) No homogenate (●), 3 μg of homogenate (▲), 6 μg of homogenate (○), or 9 μg of homogenate (△) was added to each sample.

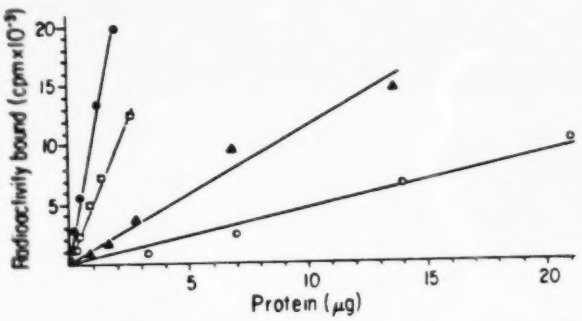

FIG. 5. Proportionality between amount of synapsin I, determined by standard assay procedure, and amount of protein in various subcellular fractions of rat brain. □, Homogenate; ▲, P_2; ⊏, SGV; and ●, peak fraction of the eluate from controlled pore glass chromatography.

at 0–4°C, the loss of sensitivity did not exceed 30–50%. Due to these variations, a calibration curve of standards was included on each sheet along with the samples to be estimated.

DISCUSSION

The data show that nitrocellulose membrane filters can be used as a convenient solid support for quantitative immunoassays of proteins. Large numbers of samples can be assayed by this procedure. For instance, in a study of the binding of synapsin I to rat brain synaptic vesicles (ref. 15 and unpublished data), 500 samples were routinely measured in 1–2 days, the most time-consuming step being the application of the samples to the sheets. It is interesting to compare the dot-immunobinding assay for synapsin I with the radioimmunoassay for this protein published previously (16). The dot-assay offers at least comparable sensitivity and range of linearity and has the considerable advantage that it does not require iodination of the antigen.

The applicability of the method is limited by the fact that not all proteins adsorb equally well to the membrane filter. In addition to the proteins described here, we tested several proteins and peptides, including protein III [a phosphoprotein-doublet of 74,000 and 55,000 daltons (17)], DARPP 32 [*d*opamine- and *a*denosine 3′,5′-monophosphate-*r*egulated *p*hospho*p*rotein 32, an acidic phosphoprotein of 32,000 daltons (18, 19)], and vasopressin (1083 daltons). Whereas protein III gave excellent results, DARPP 32 was partially and vasopressin completely washed off during the incubation and washing steps. These data suggest that smaller molecules are less well preserved on the filters, as has already been described by others (4). In the present study fixation of the samples by acetic acid/isopropanol was sufficient. Other methods to overcome this problem may include crosslinking of the sample to nitrocellulose as described for insulin (20). The use of activated supports like diazobenzyloxymethyl-paper also yields covalent linking of the sample to the support (21). It should be noted that the antibodies used have to recognize the antigen in its denatured form. The binding of each antigen to be measured should be verified before the dot-immunobinding assay can be applied.

The quantitative dot-immunobinding assay should prove particularly useful for the quantitation of membranes and membrane proteins where the antigen is not available in pure form, making radioiodination difficult or impossible. The immunization with synaptic vesicles described here resulted in antibodies directed virtually against one protein band (possibly a group of proteins) of 36,000 daltons which is apparently highly specific for synaptic vesicles (unpublished data). However, the antiserum does not have to be specific for only one antigen, in order to quantitate the amount of membrane protein. Thus, in another set of immunizations we obtained sera with a mixture of antibodies directed against several different proteins of synaptic vesicle membranes. These sera were effective for quantitation of synaptic vesicle membrane, yielding linear standard curves. Thus, the quantitative dot-immunobinding assay can also be used to measure small amounts of membranes in certain defined systems.

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